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Animal Model for Determining the No-Effect Level of an Antimicrobial Drug on Drug Resistance in the Lactose-Fermenting Enteric Flora

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Mature beagles were fed a ground-meal diet containing 0, 2, or 10 μg of oxytetracycline per g for 44 days. The 10-μg/g diet resulted in a shift from a predominantly drug-susceptible population of enteric lactose-fermenting organisms to a multiply antibiotic-resistant population which peaked at 78% resistant organisms. Since a shift to drug-resistant organisms did not occur in the group fed 2 μg/g, the level of oxytetracycline that results in increased incidence of antibiotic resistance lies between 2 and 10 μg/g in this dog model. Rats and hamsters fed diets containing oxytetracycline (10 μg/g or greater) or dihydrostreptomycin (10 μg/g), and provided suspensions of drug-susceptible *Escherichia coli*, did not develop a population of antibiotic-resistant organisms.

In a survey conducted in 1969, 5 to 43% of the livers and kidneys examined from cattle, swine, sheep, and chickens had antibiotic residues (6). Associated with these residues are potential toxicological (allergy or hypersensitivity) and microbiological health problems. The antimicrobial drugs which appear as residues in human food of animal origin have the potential of affecting the enteric microflora. From the standpoint of setting antimicrobial drug residue tolerance levels or determining acceptable analytical method sensitivities for detecting residues, it is important to consider the no-effect level of antimicrobial drugs on the drug resistance characteristics of enteric microorganisms, especially the *Enterobacteriaceae*.

The feeding of certain antimicrobials to chickens and swine results in a population of resistant lactose-fermenting enteric organisms. However, it may be difficult to obtain a source of these animals which have low enough baseline levels of resistant lactose-fermenting enteric organisms to give meaningful results. Guinea pigs are considered unacceptable because of their normal enteric flora (9). Because of the low numbers of *Escherichia coli* found in the enteric tract of rabbits (10), they, too, would likely be unacceptable.

In 1970 Guinee (4) reported that the addition of tetracycline (TC) to the diet of rats did not result in a buildup of drug-resistant *E. coli*. He also found that the addition of TC at 2, 5, and 10 μg/g to the diet of mice resulted in an increase in the recovery rate of an implanted resistant *Salmonella typhimurium* (3). This signifies that 2 μg of TC per g was enough to establish a selective advantage for the resistant *S. typhimurium*. TC fed at 10 μg/g in the diet of swine caused an increased incidence of resistant *E. coli* (5). The oral administration of oxytetracycline (OTC) to guinea pigs resulted in high concentrations of resistant *Proteus vulgaris* or *E. coli*, organisms which are not normally present in guinea pig feces (9). Thus, levels of antimicrobial drugs of 10 μg/g or less have affected enteric bacteria.

The present studies were conducted to find a suitable small animal model in which to determine the no-effect level of antimicrobial drugs on the drug resistance characteristics of the enteric flora.

MATERIALS AND METHODS

Dog experiment. Fifteen dogs (mature beagles) were divided into three experimental groups consisting of five animals per group. Each animal was maintained in a separate cage, and experimental groups were housed in separate buildings for the duration of the experiment. The animals were fed a ground-meal ration. Two treatment groups received meal supplemented with OTC at either 2 or 10 μg/g of diet continuously, whereas the control group was fed an antibiotic-free meal. Feed and water were provided ad libitum for 44 days.

Fecal samples were collected from each animal on three occasions prior to treatment and on days 1 through 10, 13, 15, 17, 21, 23, 30, 35, and 44 of the experiment. Samples were examined for resistant coliform organisms by a comparative plate-counting technique.

Rat experiment. Twelve mature albino rats were divided into four groups of three each and fed a

ground laboratory diet. One group received antibiotic-free diet, and three groups received OTC-supplemented (10 μg/g) diets. All rats were fed continuously for 6 weeks. Additionally, a fresh suspension of drug-susceptible *E. coli* was added each day to the drinking water (10^6 organisms/ml) of one treatment group. To provide the rats with exposure to a greater variety of organisms than would normally be found in a clean-cage (not sterile) environment, or clean cage plus a single strain of *E. coli*, dirt which contained no *E. coli* was added to the cage of another treatment group. Organisms from the dirt resistant to dihydrostreptomycin (DSM), ampicillin (AP), TC, furazolidone, and sulfa were isolated from MacConkey agar plates. After 6 weeks the level of OTC in the diet of the three treatment groups was raised to 50 μg/g. Rats were fed this diet for 2 weeks. Feed and water were provided ad libitum.

Individual fecal samples were collected from rats at 1, 3, and 6 weeks after treatment with OTC at 10 μg/g of meal. Samples were also collected after the 2 weeks of treatment with OTC at 50 μg/g. Fecal samples (0.5 g) were used to determine the incidence of organisms resistant to either OTC (20 μg/ml) or DSM (20 μg/ml).

Hamster experiment. Two groups of six hamsters each were continuously fed a diet which was either antibiotic free (control) or supplemented with DSM at 10 μg/g. After 47 days OTC (10 μg/g) was also added to the diet, and a suspension of *E. coli* (10^6 organisms/ml) susceptible to the spectrum of drugs tested by the paper disk method was added to the water of one treatment group daily for 2 weeks.

Fecal samples were collected from each hamster two times prior to treatment and at 3, 12, 24, and 40 days after DSM was added to the diet. They were sampled again 2 weeks after the addition of OTC and the *E. coli* suspension. The incidence of lactose-fermenting enteric organisms resistant to either DSM (20 μg/ml) or OTC (25 μg/ml) was determined on 0.5 g of feces.

Microbiological methods. The total number of lactose-fermenting enteric organisms and lactose-fermenting enteric organisms resistant to OTC in the fecal samples was determined with an aerobic plating procedure utilizing phosphate buffer as a diluent. Tenfold dilutions of fecal material were plated on MacConkey agar plus OTC (20 μg/ml) or DSM (20 μg/ml). Counts of colonies which produced reactions and were morphologically characteristic of *E. coli* from both supplemented media were compared to the unsupplemented medium to calculate the relative percentage of OTC- or DSM-resistant organisms.

Three clones which produced reactions and were morphologically typical of *E. coli* were selected from unsupplemented media plates for each sample prior to treatment and on days 1, 17, 21, 23, 30, 36, and 44 of the dog experiment. Cultures were further tested on triple-sugar iron agar and Simmons citrate agar. Cultures giving reactions typical of *E. coli* were tested for susceptibility to AP, DSM (10 μg; in place of streptomycin), cephalothin, sulfamethoxypyridazine, colistin, chloramphenicol, furazolidone (100 μg in place of nitrofurantoin), neomycin, polymyxin B, TC, and nalidixic acid (NX). The disk technique of Bauer et al. (1) was used in this procedure.

Resistance transfer was determined on all isolates exhibiting TC and/or DSM resistance by a modification of the method of Schroeder et al. (8) utilizing an *E. coli* (K-12 F^-) mutated to a high NX resistance. Five media were used: MacConkey agar; MacConkey agar plus 25 μg of NX per ml; MacConkey agar plus 25 μg of NX, 10 μg of AP, and 10 μg of dicloxacillin per ml; MacConkey agar plus 25 μg of NX and 25 μg of DSM per ml; and MacConkey agar plus 25 μg of NX and 4 μg of TC per ml. The resistance of recipient clones was confirmed by the disk diffusion susceptibility test of Bauer et al. (1).

RESULTS

In fecal samples of dogs prior to treatment, the mean prevalence of lactose-fermenting enteric organisms resistant to OTC (20 μg/ml) was <1.0% for all three groups. In the group which received the 10-μg/g diet, the mean level of organisms in fecal material which was resistant to OTC rose to 5% by day 1 and to 20% by day 2 of treatment. Thereafter, the mean incidence of resistant organisms was never below 20%. After day 10 the mean incidence of resistant organisms remained above 40% except for two sampling intervals (days 30 and 44). A peak of 78% was attained on day 36.

At most sampling times for both the control group and the group fed 2 μg of OTC per g, the mean incidence of lactose-fermenting enteric organisms in the fecal specimens was <1.0%. At two sampling times for both the control (days 2 and 4) and 2-μg/g groups (days 1 and 15) the level of resistant organisms was >1.0%; peaks of 1.4 and 3.0% were attained by the control and 2-μg/g groups, respectively. At sampling on 12 and 13 occasions for the control and 2-μg/g groups, respectively, the mean incidence of resistant organisms was <0.1%. Many of these were below the limit of detection.

The five dogs fed 10 μg of OTC per g of meal did not respond to treatment at the same time or at the same rate (Table 1). In this group, the number of dogs which had undetectable levels of resistant lactose-fermenting enteric organisms decreased from the time treatment was initiated. Subsequent to day 13 all samples (one sample per dog per sampling interval) except two had an incidence of resistant enteric organisms of 10% or more. By day 2 of treatment, the incidence of resistant organisms was 100% in one dog. The incidence of drug resistance, in animals where resistance was detectable, did not go as high and occurred more slowly in the remaining animals. Through day 10, 37 of 49 total samples had detectable levels of resistant organisms. In 15 of the 37 samples the incidence

of resistant organisms was <10%. Beyond 10 days the incidence of resistant organisms was >10% in 38 of 40 total samples.

During treatment, for the control and 2-μg/g groups, 85% of the total samples collected (one sample per dog per sampling interval) had undetectable levels of OTC-resistant organisms. Conversely, for the same period 13% of the samples collected from the 10-μg/g group had undetectable levels of resistant organisms. All of the 13% occurred during the first 10 days.

TABLE 1. Response of dogs fed OTC in diet

Day	OTC level (μg/g of meal)	No. of samples in which lactose-fermenting enteric organisms were resistant to OTC (20 μg/ml)					Total no. tested[b]
		Undetectable[a]		<1%[a]	1.0 to 10%	>10%	
		No.	%				
0	0	7	50	5	2	0	14
	2	8	57	4	2	0	14
	10	8	57	3	3	0	14
1-5	0	18	75	2	4	0	24
	2	21	84	3	1	0	25
	10	9	36	4	2	10	25
6-10	0	22	88	2	1	0	25
	2	25	100	0	0	0	25
	10	3	13	5	4	12	24
13-21	0	19	100	0	0	0	19
	2	14	78	2	1	1	18
	10	0	0	0	0	20	20
23-44	0	16	84	3	0	0	19
	2	15	75	2	2	0	20
	10	0	0	0	2	18	20

[a] Incidence of resistance.

[b] Samples were collected from five dogs per treatment group three times prior to day 0 on days 1/10, 13, 15, 17, 21, 23, 30, 35, and 44. One sample was collected from each dog per sampling time.

The predominant resistance pattern of the isolates obtained from the animals receiving the diet supplemented with OTC at 10 μg/g was DSM-TC (Table 2). Eighty isolates were multiply resistant, and four had a single resistance. Fifteen isolates were susceptible to the spectrum of drugs tested. Seventy-six and 74% of the DSM and TC resistances, respectively, were transferable. The AP resistances were 30% transferable.

In contrast to the isolates obtained from the 10-μg/g group, all nine resistant isolates obtained from the control group during treatment were singly resistant; 84 isolates were susceptible to the drugs tested. Similarly, seven of the isolates obtained from animals receiving a diet supplemented with OTC at 2 μg/g were singly resistant, and three were multiply resistant; 86 isolates were susceptible to the antibiotics tested. Most of the observed resistance in isolates from the control and 2-μg/g groups was transferable.

Resistant organisms were not recovered in either the rat or hamster experiments (Table 3). Providing the animals with a source of susceptible organisms was not sufficient to result in detectable levels of resistant organisms in fecal material.

DISCUSSION

Two prerequisites for a model system in which to determine the no-effect level of an antimicrobial drug on drug resistance in the aerobic enteric flora are low baseline levels of resistance to a spectrum of drugs and an animal system in which a response can be observed from use of a drug known to result in an increased number of pertinent organisms with drug resistance. In this study these criteria were met. The level of resistance to OTC in the lactose-fermenting enteric flora, as detected by the plate-counting procedure, was quite low and

TABLE 2. *Antibiotic resistances in lactose-fermenting enteric isolates obtained from dogs fed OTC*

Treatment period	OTC level (μg)	No. of isolates tested	Susceptible to antibiotics[a]	Antibiotic				
				AP	DSM	SU	TC	Other
Pretreatment	0	15	13	0	2	0	1	0
	2	13	11	0	3	3	0	0
	10	13	13	0	0	0	0	0
During treatment	0	93	84	0	4	0	1	4
	2	96	86	0	8	2	3	2
	10	98	15	14	81	16	80	6

[a] All isolates were tested with paper disks impregnated with the following antibiotics (one antibiotic per paper disk): AP (10 μg); DSM (10 μg); cephalothin (30 μg); sulfamethoxypyridazine (SU), (250 μg); colistin (10 μg); chloramphenicol (30 μg); furazolidone (100 μg); neomycin (30 μg); TC (30 μg); and NX (30 μg).

TABLE 3. *Emergence of antibiotic resistance in three animal species after feeding diets supplemented with either OTC or DSM*

Animal	Antibiotic/ diet (μg/g)	Days of treatment	Highest percentage of lactose fermenting enteric organisms resistant to:[a]	
			OTC	DSM
Rat	OTC (10)	42	0	0
Rat	OTC (50)	14	0	
Hamster	DSM (10)	40		0
Hamster	OTC (10)	21	0	0
Dog	OTC (10)	44	78	
Dog	OTC (2)	44	3	

[a] Values are group means. Zeros indicate undetectable levels of lactose-fermenting enteric organisms resistant to the specified antibiotic. Pretreatment incidences of resistant organisms were <1.0% for the dogs.

remained so in the control animals. The incidence of TC resistance in the few isolates tested prior to treatment was also quite low. The incidence of resistance to DSM and sulfonamide in these isolates was higher than the incidence of resistance to TC, but low enough to detect changes.

The results indicate the no-effect level on drug resistance in lactose-fermenting enteric bacteria for OTC in the diet used in the dog model of this study lies between 2 and 10 μg/g. As can be observed in Table 1, length of exposure is related to magnitude of response in some animals, but a large response can occur in a very short time in other animals. This may be related to individual baseline levels of resistant organisms in the gastrointestinal tract or to genetic differences of the resident flora.

The lack of response in the animals fed the diet containing 2 μg of OTC per g may well be related to the minimal inhibitory concentrations of antibiotics on the organisms. It may also be related to drug absorption, drug inactivation (protein binding or chelation), drug stability, or dilution effects due to water consumption and internal secretions. Time may be another factor. Exposure over a much longer time may affect either the incidence of organisms resistant to TC or another drug such as DSM.

In determining the no-effect level of an antimicrobial on the enteric flora, homologous resistance to the drug being studied, as well as heterologous resistance, should be determined. In this study resistance to AP, DSM, and sulfonamide increased, as did resistance to the homologous drug. This aspect has been reported previously (2, 7), but the levels of drugs initiating the response were higher than those ordinarily found as residues in food.

This study confirms those previously reported (4) in that the oral administration of TC does not result in the emergence of a population of resistant *E. coli* in the rat. Even by adding a suspension of susceptible *E. coli* to the drinking water of the rats and hamsters, they both proved to be unsatisfactory as a small animal model for determining the no-effect level of an antimicrobial on the enteric flora. However, the particular strain of *E. coli* used in these studies may have been refractory to becoming drug resistant. In comparison, the dog model was found to be acceptable.

The no-effect level on drug resistance may vary among the various animal species in which a response to antimicrobials can be noted in the lactose-fermenting enteric flora. The dog model reported here is susceptible to at least 10 μg/g of diet (perhaps less) for OTC. Other animal species may be more susceptible. However, the availability of dogs with low baselines of resistant enteric organisms and their sensitivity for determining the no-effect level makes this model acceptable. This model is acceptable only for determining which antimicrobial drugs may produce an effect in the human if consumed as residues in food items. If human studies are considered necessary, the dog model may indicate the range of dose levels necessary. Due to species differences, direct extrapolation to humans should not be considered. Species differences may influence some drugs more than others.

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